KSÜ Tarım ve Doğa Derg 25 (6): 1315-1325, 2022
KSU J. Agric Nat 25 (6): 1315-1325, 2022
https://doi.org/10.18016/ksutarimdoga.vi.993825

Investigating *In Vitro* Genotoxic Effects of Sweetener Xylitol

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ABSTRACT
Xylitol (XYL) is a sweetener used as a food additive in the food industry. In the present study, four different genotoxicity assays (chromosomal aberration=CA, sister chromatid exchange=SCE, cytokinesis-block micronucleus cytome=CBMN-Cyt, and comet assays) were conducted to assess the potential genotoxicity of XYL in human lymphocytes. Four concentrations (125, 250, 500, and 1000 µg mL^{-1}) of XYL were applied to lymphocytes obtained from three healthy young donors. The frequency of CA was not significantly affected by 24-h administration of XYL (except 1000 µg mL^{-1} for the number of CAs). 48 h treatment of XYL increased the frequency of CAs and abnormal cells. However, this increase was significant at only two highest concentrations. XYL significantly increased SCE/cell rate at the two highest concentrations at both treatment times (24 and 48h). Similarly, the frequency of MN, Nuclear buds (NBUDs), and Nucleoplasmic bridges (NPBs) significantly increased by XYL only at the high concentrations. It raised the comet parameters at the two highest concentrations. These observations showed that XYL, only at high concentrations, may have a genotoxic effect on human lymphocytes *in vitro*. For this reason, it can be concluded that its use at low concentrations may not cause DNA or chromosomal damage.

Genetic

Research Article

Article History
Received : 10.09.2021
Accepted : 26.11.2021

Keywords
Genotoxicity
Human lymphocytes
Sweetener
Xylitol

Tatlandırıcı Olan Xylitol'ün *In Vitro* Genotoksik Etkilerinin İncelenmesi

ÖZET
Xylitol (XYL), gıda endüstrisinde gıda katkı maddesi olarak kullanılan bir tatlandırıcıdır. Bu çalışmada, insan lenfositlerinde XYL'nin potansiyel genotoksisitesini değerlendirmek için dört farklı genotoksisite testi (kromozomal anormallik=KA, kardeş kromatid değişimi=KKD, sitokinez-blok mikronükleus sitom=CBMN-Cyt ve comet testleri) gerçekleştirilmiştir. Genç ve sağlıklı donörlerden elde edilen lenfositlere dört farklı konsantrasyonda (125, 250, 500 ve 1000 µg mL^{-1}) XYL uygulanmıştır. KA frekansı, 24 saatlik XYL uygulamasından önemli ölçüde etkilenmemiştir (anormal hücre sayısı için 1000 µg mL^{-1} hariç). 48 saatlik XYL muamelesi, kromozomal anormalliklerin ve anormal hücrelerin sıklığını arttırmıştır. Ancak bu artış en yüksek iki konsantrasyonda anlamlıdır. XYL, tüm uygulama sürelerinde (24 ve 48 saat) en yüksek iki konsantrasyonda KKD/hücre oranını önemli ölçüde yükseltmiştir. Benzer şekilde, MN, Nükleer tomurcuklar (NBUD'ler) ve Nükleoplazmik köprülerin (NPB'ler) sıklığı, XYL'ün yüksek konsantrasyonlarında anlamlı artış göstermiştir. XYL comet parametrelerini en yüksek iki konsantrasyonda yükseltmiştir. Bu gözlemler, XYL'nin *in vitro* insan lenfositlerinde yalnızca yüksek konsantrasyonlarda genotoksik bir etkiye sahip olabileceğini göstermiştir. Bu nedenle düşük konsantrasyonlarda kullanımının DNA veya kromozomal hasara neden olmayabileceği düşünülmektedir.

Genetik

Araştırma Makalesi

Makale Tarihçesi
Geliş Tarihi : 10.09.2021
Kabul Tarihi : 26.11.2021

Anahtar Kelimeler
Genotoksisite
İnsan lenfositleri
Tatlandırıcı
Xylitol

Atıf Şekli: Avuloglu-Yilmaz E, Yuzbasioglu D, Unal F 2022. Tatlandırıcı Olan Xylitol'ün *In Vitro* Genotoksik Etkilerinin İncelenmesi. KSÜ Tarım ve Doğa Derg 25 (6): 1315-1325. https://doi.org/10.18016/ksutarimdoga.vi.993825
To Cite : Avuloglu-Yilmaz E, Yuzbasioglu D, Unal F 2022. Investigating *In Vitro* Genotoxic Effects of Sweetener Xylitol. KSU J. Agric Nat 25 (6): 1315-1325. https://doi.org/10.18016/ksutarimdoga.vi.993825

INTRODUCTION

Food additives are substances that are supplemented in the production process to improve the characteristics of foods such as color, taste, smell, nutritional value, and shelf life. Retrospective studies on the currently used food additives have shown that some of them may have adverse effects on human health (Gultekin et al., 2017; Ali et al., 2020; Cadirci et al., 2020).

The use of several additives (artificial sweeteners, food colorings, flavor enhancers, etc.) causes an intense debate on their adverse effects. It is thought to have a higher risk than the benefit arising from the use of food additives. It was found in a study that consumers do not want to buy processed foods containing food additives due to potential hazards such as cancer, asthma, digestive problems, neurological problems, heart disease, obesity, and allergies (Shim et al., 2011; Yin et al., 2020). Naturalness is seen as a major feature for consumers. While natural foods are thought to be healthy and safe, unnatural foods are considered to carry the risk of disease (Kadim, 2017). In studies, supporting this situation, it has been determined that some food additives have genotoxic and carcinogenic effects. Others have also been shown to play a role in the formation of hyperactivity, neurodegenerative diseases, allergies, diabetes, obesity, reproduction, and disorders related to the gastrointestinal tract. For these reasons, studies examining the effects of these substances on health have increased and restrictions have been placed on the use of these substances (Yuzbasioglu et al., 2014; Comert and Gudek, 2017; Hassan and Jasem, 2020).

The International Food Codex Commission (CAC), formed by the World Health Organization (WHO) and the Food Agriculture Organization (FAO), defines sweetener as a "non-sugar substance that gives a sweet taste to food". Substances with sweetening properties show different physical, chemical, and physicochemical characteristics due to their different chemical structures. The oldest substance known for its sweetening effect is sucrose in the sugar group, and glucose, fructose, hydrolysed starch syrups, and high fructose corn syrups are natural ingredients of agricultural origin that provide sweetness in foods. However, as can be understood from the definition of CAC given above, these substances with sugar properties are not considered food additives. Sweeteners are food additives substituted for sugar, with a sweet taste similar to sugar, but with significantly less energy. Sweeteners can be divided into three groups as sugar alcohols (polyols), artificial sweeteners, and natural sweeteners (Kızılaslan, 2017; Yin et al., 2020). Polyols (sugar alcohols) are the most commonly used sweetener group and are natural sugar replacers. Polyols do not require insulin to enter the cells, therefore, they are suitable for use by diabetics. However, when the intake is above 50-60 g/daily, the excess amount turns into glucose in the liver. Xylitol (XYL) an approved additive (E 967), and hydrogenated form of monosaccharides, is tolerated like other polyols, but its excessive consumption can cause a laxative effect. The EU Food Scientific Committee indicated that the daily consumption of polyols, other than sensitive individuals, should be less than 20 g, otherwise it is likely to cause a laxative effect. XYL is used in chewing gum, peppermint, acacia gum, lozenges, and other confectionery. It is also used as an excipient in toothpaste, other special dental products, and pharmaceutical products (Mäkinen, 2016; Wölnerhanssen et al., 2020). While sweeteners have been positively welcomed by some consumers, some others have been cautious approaches to these molecules. Sweeteners have varied over the past years and their usage has increased during the last 40 years. Therefore, a large mass of people is consuming foods containing sweeteners (Lemos et al., 2018).

Since it is understood that agents with genotoxic effects can cause health problems, short-term genotoxicity assays have been developed and used to detect these agents. Nowadays, genotoxicity assays are widely used as a biomarker to assess the genotoxic potential of food additives. The most commonly used biomarkers are chromosomal aberration (CA), sister chromatid exchange (SCE), cytokinesis block micronucleus cytome (CBMN-Cyt), and comet assays (Lal et al., 2019; Trompowsky et al., 2019; Gooderham et al., 2020).

CA test is used to detect structural and numerical aberrations. Therefore, it is effective in detecting both the clastogenic and aneugenic effects of the test substance. Chemicals that directly induce DNA strand breaks can induce chromatid-type aberrations in the G2 stage of the cell cycle and they produce chromosome-type aberrations in G0 and G1 stages. Most chemical mutagens produce S-phase-associated lesions leading to chromosomal damage, resulting in chromatid-type aberrations more frequently. Since the formation of SCE is directly related to DNA replication in human cells exposed to mutagens in the G2 phase of the cell cycle, the frequency of SCE may

only increase after the subsequent replication cycle. Homologous recombination between sister chromatids is considered to be the main mechanism for SCE formation. This mechanism is considered to be protected because in general, no DNA information is exchanged by homologous recombination (Mourelatos, 1996; Zhang, 2013; Mourelatos, 2016; Yadav et al., 2019; Kadlcikova et al., 2020).

The mitotic index shows the ratio of cells undergoing mitosis, while the replication index evaluates how many mitotic cells have completed one, two, three, or more consecutive cell cycles. The decrease in the mitotic index indicates a cytotoxic effect, while the decrease in the replication index reveals a delay in the cellular proliferation kinetics induced by various chemicals (Hemachandra and Pathiratne, 2016). The inhibition of mitochondrial function is thought to be the cause of the decrease in the mitotic index (Azab et al., 2017). This parameter is a useful biomarker for the evaluation of cytotoxic and cytostatic activities of chemicals in tumor and normal experimental models. Chemicals affecting mitochondrial metabolism can alter energy production and create oxidative stress and eventually inhibit cytochrome 1A1 (CYP1A1) activity. These effects threaten cellular homeostasis and alter cellular proliferation capacity (Álvarez-Barrera et. al., 2017; Erikel et al., 2019; Cavalcanti et al., 2020).

Micronuclei (MNi) consist of a chromosome loss or breakage. While nucleoplasmic bridges (NPBs) originated from DNA strand break or telomere end fusions, nuclear buds (NBUDs) are formed due to the amplified DNA or elimination of DNA repair complexes (Fenech, 2006 and 2007; Gundogan et al., 2018). The cells carrying these three abnormalities are called chromosomally unstable cells. These types of aberrations are common in cancer cells. Chromosomal instability (CI) causes changes in gene dosage, the rapid growth of a cell, and mutation. This causes the cell to escape from the homeostatic control mechanisms due to its genetic plasticity and thus become immortalized and transform into various abnormal genotypes. All these variations make tumor cells survive and escape from the immune system (Negrini et al., 2010; Ruiz-Ruiz et al., 2020).

Depending on the technique used (neutral, alkali, etc.), the comet assay shows single and double-strand breaks. The breaks are associated with chromosomal abnormalities and genomic instability. Genomic instability is directly related to malignancy (Azqueta and Collins, 2013; Vodicka et al., 2019; Souto et al., 2020).

The genotoxicity of XYL was evaluated by JECFA (1978) and it was stated that it is not genotoxic. However, no other genotoxicity study of xylitol with human lymphocytes was available. Therefore, the main goal of the present study is to investigate the possible genotoxicity of sweetener xylitol using CA, SCE, CBMN-Cyt, and comet assays on human peripheral lymphocytes. Besides, the effect of XYL on the replication index (RI), mitotic index (MI), and nuclear division index (NDI) was also determined.

MATERIAL and METHODS

Cultured lymphocytes were used for CA, SCE, and CBMN-Cyt assays, and isolated lymphocytes were used for the comet assay. Peripheral blood was obtained from three healthy young (aged 23-27, two women, and one male) donors. The study was carried out with the permission of the ethical committee of the Faculty of Medicine, Gazi University (No: 234; 05.08.2017).

The molecular weight of the test material Xylitol (Cas number: 87-99-0) is 152.15 g mol^{-1}, its molecular formula is $C_5H_{12}O_5$ and obtained from Sigma-Aldrich (purity: ≥ 0.99). JECFA has not specified the ADI (Acceptable Daily Intake) value of XYL (ADI Not Specified) and the amounts used in humans are not clear. Therefore, the LD_{50} value in rodents was considered as a reference to determine appropriate dose ranges (Anonymous, 2021). Firstly, 31.25, 62.50, 125, 250, 500, 1000, and 2000 µg mL^{-1} were tested using cell-proliferating activity/mitotic index, in a preliminary study. Depending on these results, 125, 250, 500, and 1000 µg mL^{-1} were chosen as the test concentrations. Xylitol was dissolved in distilled water.

Chromosomal aberrations and sister chromatid exchange assay

To determine CAs, the method of Evans' (1984) was applied with some modifications (Yuzbasioglu et al., 2006). SCE assay was carried out using the method of Perry and Wolff (1974) with some alterations (Speit and Haupter, 1985). For these tests, 200 µL of heparinized whole blood was added immediately to culture tubes containing Chromosome Medium B. Bromodeoxyuridine (BrdU, 10 µg mL^{-1}) was supplemented into the same tubes and then incubated at 37°C for 72 h. Cells were treated with XYL for the last 24 and 48 hours of the culture. On the other hand, distilled water and 0.20 µg mL^{-1} mitomycin-C (MMC) was added to the experimental setup as the negative and positive control, respectively. MMC is an agent recommended by the OECD (test number 473 and 487) as a positive control in test guidelines (OECD 2016a and OECD 2016b). At the 70th hour of the culture, colchicine (0.06 µg mL^{-1}) was supplemented to each tube to block mitosis. The culture was terminated at the 72nd hour. Cells were centrifuged and treated with 0.075 M KCl. Lymphocytes were fixed in cold 3: 1 methanol: acetic acid three times and then the cell suspension was dropped onto previously cleaned cold slides. The

slides were stained in 5% Giemsa. A total of 300 metaphases for each concentration (100 metaphases per donor) were analyzed to detect CAs. To determine SCEs, a total of twenty-five cells at second metaphases were evaluated per donor (a total of 75-second metaphases for each concentration). Also, the MI was assessed by scoring a total of 3000 cells (1000 cells/donor). 100 cells per donor were scored to determine the RI (totally 300 cells/concentration). RI was calculated using the following formula: RI = [M1 + 2xM2 + 3xM3]/N, where M1, M2, and M3 represent the number of cells undergoing first, second and third mitosis and N is the total number of metaphases scored (Schneider and Lewis,1981).

Cytokinesis-block micronucleus cytome assay

The procedure of Fenech (2007) was used with some alterations for the CBMN-Cyt assay. 200 µL of blood specimen was added straightaway to tubes containing the Chromosome Medium B to cultivate lymphocytes for 72 h at 37°C. Cells were treated with four concentrations of XYL (125, 250, 500, and 1000 µg mL^{-1}) for the last 48 hours. A negative (distilled water) and positive control (MMC, 0.20 µg mL^{-1}) were also maintained. To prevent cytokinesis, 5.2 µg mL^{-1} of Cytochalasin-B was added to the culture at 44th h. Then the cultures were centrifuged and the supernatant was removed. Following the addition of cold KCl, suspensions were kept in the refrigerator (+4°C) for 5 min. The tubes were centrifuged and the supernatant was removed. Cells were fixed in cold 3: 1 methanol: acetic acid for 15 minutes in the refrigerator. Fixation was repeated twice. Formaldehyde was added to the final fixative and centrifuged for the last time. The supernatant was discarded and the remaining cell suspension was homogenized using a pipette. The suspension was spread over the previously cleaned cold slides and left at room temperature for 24 hours to dry.

Comet assay

The technique used by Singh et al. (1988) was applied with some alterations. Lymphocytes were isolated from whole blood samples using biocoll. Cell viability of lymphocytes was determined to be ≥ 97% using trypan blue. Lymphocytes were incubated with four concentrations of XYL (125, 250, 500, and 1000 µg mL^{-1}) for 1 h at 37°C. A positive (100 µM H_2O_2) and negative control were also used. Following treatment, the supernatant was removed by centrifugation and resuspended with PBS. Lymphocytes were mixed with low melting heat agar and spread on slides coated with high melting heat agar. After lysis, slides were kept in electrophoresis buffer and electrophoresed at 300 mA, 25 V for 20 minutes. Afterward, slides were kept in 0.4 M Tris buffer (pH = 7.5) and stained with EtBr. Totally 300 cells (100 cells per donor) for each concentration were examined under a fluorescent microscope using "Comet Assay IV", Perceptive Instruments Ltd., UK analysis system. The degree of damage to the cells was evaluated using comet tail length (µm), tail intensity (%), and tail moment.

Statistical analysis

To reveal the dose-effect relationship for the mitotic index, replication index, SCE / cell, abnormal cell, CA/cell, MN/cell, NBUDs/cell, NPBs/cell, nuclear division index, tail length, tail intensity, and tail moment regression analysis was applied using SPSS 22.0 program. Mitotic index (MI), replication index (RI), abnormal cell frequency, CA/cell, MN/cell, NBUDs/cell, NPBs/cell, and nuclear division index (NDI) results obtained from the experimental and control groups were analyzed using z-test, sister chromatid exchange and comet assay results were analyzed using t-test.

RESULTS

XYL increased the frequency of CAs and CA/cell in human lymphocytes at both treatment periods (Table 1). However, increases were not significant compared to the negative control at 24 h (except 1000 µg mL^{-1} %CAs). At 48 h treatment, increases were significant and concentration-dependent at the two highest concentrations (r=0.99 for both CAs and CAs/cell). Following XYL exposure, six types of structural and one type of numerical (polyploidy) aberrations have been detected in human lymphocytes. Structural abnormalities are chromatid (63.37%) and chromosome breaks (12.98%), sister chromatid union (13.74%), dicentric chromosome (7.63%), fragment (0.76%), and chromatid exchange (0.76%). The chromatid break was the most common abnormality observed.

The effect of XYL on SCE/cell ratio and RI at 24 h and 48 h exposures were presented in Table 2. XYL has raised the ratio of SCE/cell at both treatment periods (except 125 µg mL^{-1} at 24 h). Increasing was significant at the two highest concentrations and concentration-dependent (r=0.90 and r=0.93 at 24 h and 48 h, respectively). XYL produced up to 10 SCEs/Cell in lymphocytes. MI significantly decreased at all the applied concentrations of XYL at 48h treatments, however the reduction was significant at the only two highest concentrations at 24h applications (Figure 1). Decreasing in the MI was concentration-dependent at both treatment periods (r=-0.99 and r=-0.93, at 24h and 48h, respectively). On the contrary, XYL did not significantly affect the RI (Table 2).

In this study, XYL has been evaluated using CBMN-Cyt assay and determined that the frequencies of MN, NBUDs, and NPBs increased in a concentration-

dependent manner (r= 0.91, r=0.98, r=0.90, respectively). However, this increase was significant only at the concentrations of 500 and 1000 µg mL^{-1} for the frequency of MN and NBDs, and at the highest concentration for the frequency of NPBs over the negative control (Table 3). On the other hand, XYL did not affect NDI.

XYL has also been assessed using comet assay and observed that XYL significantly increased the tail length, tail intensity, and tail moment at the two highest concentrations in a concentration-dependent manner (respectively r=0.99, r=0.82, and r=0.86) in human lymphocytes (Figure 2-4).

Çizelge 1. XYL'ün insan lenfositlerinde kromozom anormallik oranına etkisi
Table 1. Effect of XYL on the frequency of chromosome aberrations in human lymphocytes.

Test subs.	Treatment h	Conc. mg mL^{-1}	Aberrations ctb	csb	scu	dic	ce	f	p	Abnormal cell ± SE (%)	CA/cell ± SE
Control	24	0	9	-	-	-	-	-	-	3.00±0.985	0.030±0.009
MMC	24	0.20	25	10	2	6	3	-	-	14.67±2.042	0.153±0.021
XYL	24	125	8	5	2	-	-	-	-	5.00±1.260	0.050±0.013
		250	7	1	6	2	-	-	-	5.33±1.300	0.053±0.013
		500	12	2	1	3	-	-	-	6.00±1.370	0.060±0.014
		1000	12	5	2	1	-	-	1	5.67±1.340	0.067±0.014*
Control	48	0	3	2	1	-	-	-	-	2.00±0.081	0.020±0.008
MMC	48	0.20	31	7	7	2	5	-	-	16.67±2.152	0.173±0.022
XYL	48	125	6	1	1	-	-	-	-	2.67±0.930	0.027±0.009
		250	10	1	1	1	-	-	-	4.33±1.175	0.043±0.012
		500	14	1	2	-	-	-	-	5.67±1.340*	0.057±0.013*
		1000	14	1	3	3	1	1	-	6.67±1.440**	0.077±0.015**
Fab (%)			63.37	12.98	13.74	7.63	0.76	0.76	0.76		

Test subs: Test substance, h: hour, Conc: Concentrations, ctb: Chromatid break, csb: chromosome break, scu: sister chromatid union, dic: dicentric chromosome, ce: chromatid exchange, f: fragment, p: polyploidy, Fab: Frequency of abnormalities, SE: Standart Error, *Significantly different from the control $p < 0.05$ (z test), **Significantly different from the control $p < 0.01$ (z test).

Çizelge 2. XYL'ün insan lenfositlerinde SCE ve RI üzerindeki etkisi
Table 2. Effect of XYL on SCE frequencies and RI in human lymphocytes

Test subs.	Treatment h	Conc. mg mL^{-1}	Min-max SCE	SCE/cell ± SE	M_1	M_2	M_3	RI ± SE
Control	24	0	1-7	2.76±0.169	96	54	150	2.18±0.068
MMC	24	0.20	17-38	27.99±0.611	105	75	120	2.05±0.061
XYL	24	125	1-6	2.75±0.172	81	72	147	2.22±0.071
		250	1-7	2.84±0.177	74	77	149	2.25±0.072
		500	2-10	3.75±0.193*	112	66	122	2.03±0.060
		1000	1-9	4.61±0.210*	111	62	127	2.05±0.061
Control	48	0	1-6	2.01±0.141	112	80	108	1.99±0.057
MMC	48	0.20	20-42	27.88±0.537	118	54	128	2.03±0.060
XYL	48	125	1-6	2.28±0,138	111	73	116	2.02±0.059
		250	1-6	2.28±0,136	114	86	100	1.95±0.055
		500	1-7	3.41±0,143*	110	72	118	2.03±0.060
		1000	1-7	3.67±0,184*	90	78	132	2.14±0.066

Test subs: Test substance, h: hour, Conc: Concentrations, SE: Standart error, * Significantly different from the control $p<0.05$ (t-test).

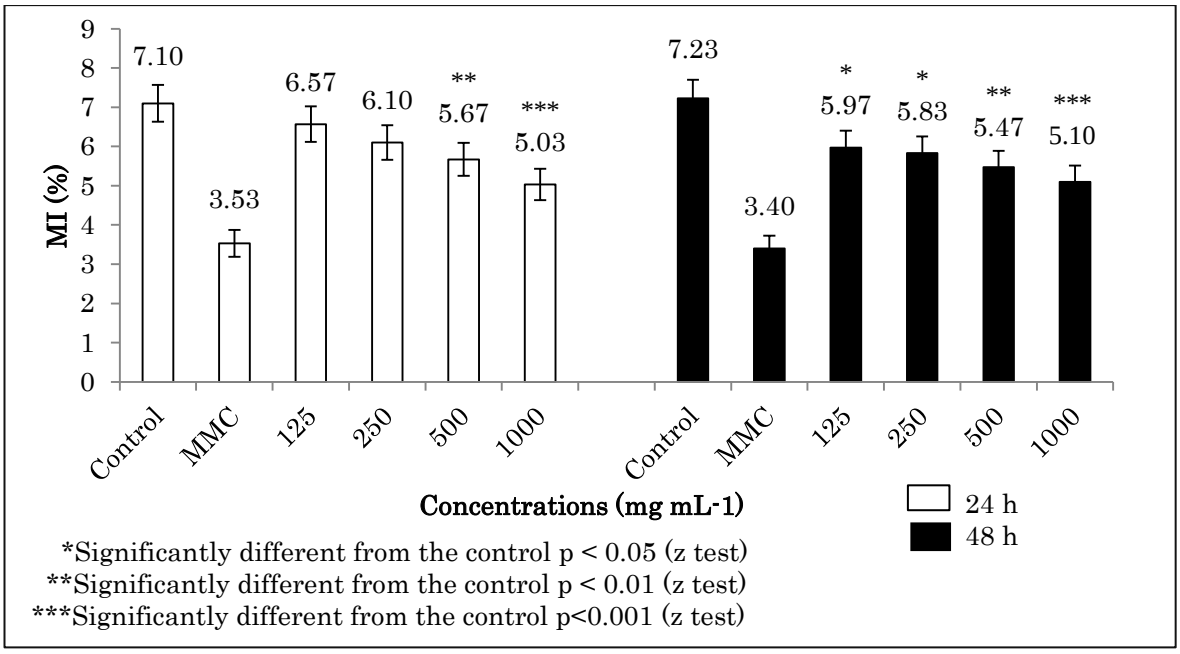

Figure 1. Effect of XYL on MI in human lymphocytes
Şekil 1. XYL'ün insan lenfositlerinde MI'e etkisi

Çizelge 3. XYL'ün insan lenfositlerinde MN, NBUD, NPB frekansı ve NDI üzerindeki etkisi
Table 3. Effect of XYL on the frequency of MN, NBUD, NPB, and NDI in human lymphocytes

Test subs.	Treatment h	Conc. mg mL^{-1}	Number of MN/BN cell (1)	(2)	MN ± SE (%)	Nuclear division index (NDI) ± SE	Nuclear bud (NBUD)± SE (%)	Nucleoplasmic bridge (NPB) ± SE (%)
Control	48	0	15	-	0.00 ± 0.13	1.42 ± 0.31	0.17 ± 0.08	0.03 ± 0.03
MMC	48	0.20	72	4	2.67 ± 0.29	1.31 ± 0.29	0.83 ± 0.17	0.33 ± 0.11
XYL	48	125	12	-	0.40 ± 0.12	1.36 ± 0.30	0.23 ± 0.08	0.03 ± 0.03
		250	19	3	0.83 ± 0.17	1.35 ± 0.30	0.33 ± 0.10	0.07 ± 0.05
		500	28	-	0.93 ± 0.18*	1.35 ± 0.30	0.47 ± 0.12*	0.10 ± 0.06
		1000	30	-	1.00 ± 0.18*	1.37 ± 0.30	0.50 ± 0.13*	0.23 ± 0.09*

Test subs: Test substance, h: hour, Conc: Concentrations, SE: Standart error, *Significantly different from the control p<0.05 (z-test).

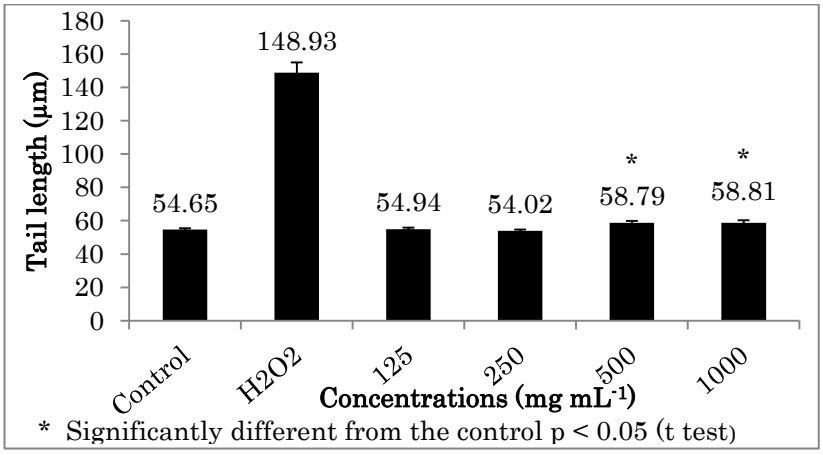

Figure 2. Comet tail length after the treatment of XYL in isolated human lymphocytes
Şekil 2. İzole insan lenfositlerinde XYL muamelesinden sonra comet kuyruğu uzunluğu

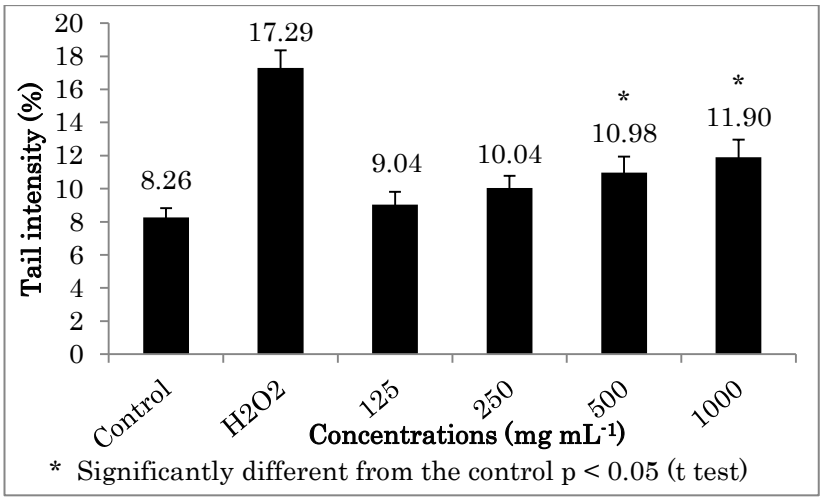

Figure 3. Comet tail intensity after the treatment of XYL in isolated human lymphocytes
Şekil 3. İzole insan lenfositlerinde XYL muamelesinden sonra comet kuyruk yoğunluğu

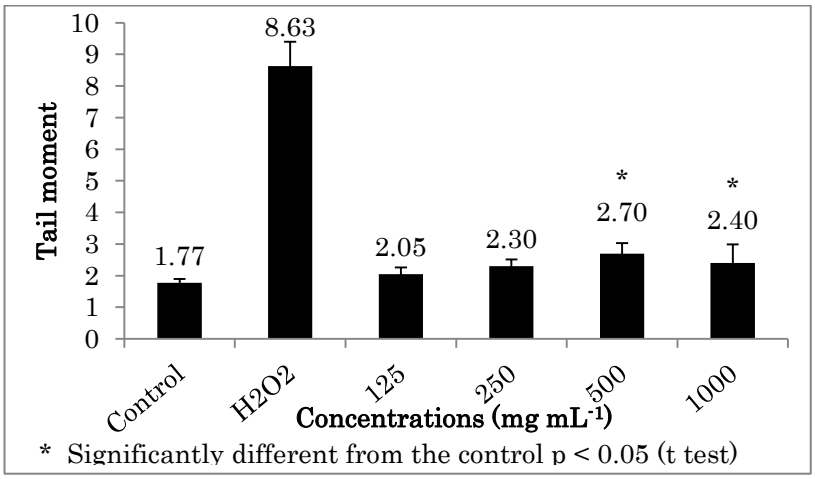

Figure 4. Comet tail moment after the treatment of XYL in isolated human lymphocytes
Şekil 4. İzole insan lenfositlerinde XYL muamelesinden sonra comet kuyruk momenti

DISCUSSION and CONCLUSIONS

To improve the appearance and flavors of the foods offered or to be consumed to the consumer's desire and/or to prevent their spoilage and extend their shelf life, the ingredients added to the foods are called food additives. In the last 30 years, especially in developed countries, there has been an increase in additives used in foods. Their increasing results from the production techniques brought by the developing technology and the diversity of consumer taste. Today, while people maintain to access durable and rich food sources, their concerns about food safety are regularly increasing (Amin et al., 2013; Khan et al., 2020). For this reason, any risk assessment should be considered to protect human health.

Physical and chemical agents interacting with DNA and DNA-related cellular components (mitotic and meiotic spindle threads, replication enzymes, DNA repair system enzymes, proteins controlling the cell cycle, genes associated with apoptosis, and proteins providing defense against oxidative damage), and cause damage to the genome are called as genotoxic (Bellani et al., 2020). Genotoxic agents that people are exposed to may cause tissue damage, infertility, birth defects, cancer, and some genetic and multifactorial diseases both at the individual level and in the future generations. It has been determined that there is a strong relationship between genotoxicity and cancer, and many compounds that are carcinogenic to humans are also genotoxic. Genotoxic agents have been found to have some adverse health effects. Therefore, short-term genotoxicity assays have been developed to determine genotoxic agents. These tests that can be used routinely *in vivo* or *in vitro* and accepted as valid by international agencies are widely applied in determining the genotoxic potency of food additives (Bastaki et al., 2017; Otabe et al., 2019; Avuloglu-Yilmaz et al., 2020; Bellani et al., 2020; Khan et al., 2020).

This research revealed that XYL induced the incidence of CAs, SCEs, MN, NBUD, NPB, and DNA damage only at the higher concentrations. The

highest rate of chromatid breaks (63.37%) and the increased frequency of CA at high concentrations in both 24 and 48 hours of treatment suggest that XYL may have affected the G2 stage. The increased frequency of SCE at the highest concentrations indicates that the chemical may also influence the DNA replication and G2 phase. Again, for the same concentrations, increased MN, NBUD and NPBs may be indicative of a clastogenic and/or aneugenic effect, and chromosomal instability. The results of all genotoxicity tests used in this study support each other.

According to the report published by JECFA (1978), the following test procedures were performed on XYL. Ames test with TA 1535, TA 1537, and TA 1538 strains of *Salmonella typhimurium* with and without metabolic activation, host-mediated assay in the mouse with *Salmonella typhimurium* TA1530, TA 1532, and TA 1964 strains, micronucleus assay with Fullinsdorf albino mice, and chromosomal aberration test in cultured human lymphocytes. It was reported that XYL did not cause observable mutagenic effects in any of these test systems (JECFA, 1978). However, the report did not contain important information such as concentration and treatment time used in the tests. As far as we know, there was no comprehensive available study on the possible genotoxicity of XYL in human lymphocytes. On the other hand, numerous studies are investigating the genotoxic effects of some sweeteners in different cell groups. For example, maltitol, sugar alcohol such as xylitol, did not stimulate SCEs in human peripheral lymphocytes at applied concentrations (1.25, 2.5, and 5 mg mL^{-1}) and durations (24 and 48 hours) used. Chromosomal abnormalities increased but this increase was not statistically significant. On the other hand, maltitol increased the frequency of MN at 24 and 48 hours, but not in a concentration-dependent manner. Maltitol did not affect the RI and MI at all the applications. As a result, it has been interpreted that maltitol has genotoxic potential but this potential effect is weak and it is not cytotoxic (Canımoglu and Rencuzogullari, 2006). The same researchers evaluated the genotoxic effect of maltitol in the bone marrow cells of rats. They reported that maltitol (2.5, 5, and 10 g kg^{-1}, 6, 12, and 24 hours) did not affect the frequency of chromosome aberrations and mitotic index. Maltitol was also administered intraperitoneally to pregnant rats during the first 7 days of pregnancy (1st trimester) to determine teratogenic and embryotoxic effects. While maltitol did not reveal a teratogenic effect, it reduced the weight of fetuses and caused growth retardation at the highest dose (4 g kg^{-1}) (Canımoglu and Rencuzogullari, 2013). In contrast to the previous two studies, the present study indicated that xylitol may be genotoxic, but only at high concentrations. This discrepancy might result from the differences in concentrations used. Though these two chemicals are in the same group, they may have different genotoxic effects that might be the reason for the difference.

The potential genotoxicity of erythritol was investigated by short-term assays. Ames test was applied to the strains of *Salmonella typhimurium* TA98, TA1537 (detects mutagens that cause frameshift), TA100, TA1535, and *Escherichia coli* WP2 uvrA (detects base pair changes), but no positive result was detected. *In vitro* CA test with CHL cells and *in vitro* micronucleus assay with L5178Y tk +/- cells, no mutagenicity was observed at the concentrations used (1250, 2500, and 5000 g mL^{-1}). Again, in the comet assay, using the same concentrations of erythritol in L5178Y tk +/- cells, a significant rise in DNA damage was detected at higher concentrations. Besides, the *in vivo* micronucleus test carried out on bone marrow cells of male ICR mice indicated that oral erythritol administration (1250, 2500, and 5000 mg kg^{-1}) did not increase MN formation. When all these results are taken into consideration, it has been stated that erythritol has no mutagenic and chromosome damaging effects (Chung and Lee, 2013).

In a study investigating the effect of maternal sorbitol intake on offspring, sorbitol was given to Wistar rat offspring for 14 days via breast milk. Administered doses were 0.15, 1.5 and 150 mg kg^{-1}. Significant changes in some biochemical parameters such as ALT, AST, and LDLc were observed in offspring. Sorbitol exposure triggered hepatocyte genotoxicity, including micronucleus induction. Comet analysis of blood cells revealed dose-dependent genotoxic effects in offspring exposed to sorbitol. The authors concluded that significant metabolic changes and genotoxic responses could be induced in offspring exposed to sorbitol (Cardoso et al., 2016).

Aspartame is a methyl ester composed of aspartic acid and phenylalanine. It has been added to more than 6000 different products (Kirkland and Gatehouse, 2015). Therefore, aspartame was largely investigated in terms of genotoxicity and carcinogenicity. The effects of aspartame (400-1600 mg kg^{-1} day) on bone marrow and spermatogonium cells of Holtzman rats were investigated by the chromosome aberration assay. No increase in CAs was observed in doses used (Bowles, 1970). Aspartame was given to C57BL / 6 mice at doses of 15 and 150 mg kg^{-1} for five days but an increase in CA frequency was detected at any dose (Durnev et al., 1995). There was no rise in DNA migration in comet assay in the eight tissues of mice exposed to aspartame (2000 mg kg^{-1} for 3 and 24 h) (Sasaki et al., 2002). On the contrary, many studies are reporting positive results regarding aspartame. Rencuzogullari et al. (2004) investigated the possible genotoxic effects

of aspartame (500-2000 µg mL^{-1} for 24 and 48 h) in human lymphocytes using CA and MN assays. They observed a significant increment in the frequency of CAs and MN (Rencuzogullari et al., 2004). The genotoxic effect of aspartame was also investigated by MN and CA assays in the bone marrow and blood cells of Swiss albino mice (250-1000 mg kg^{-1} for 24, 48, and 72 hours). The frequency of CA and MN significantly raised especially at high concentrations and long treatment periods (Kamath et al., 2010). Increased DNA damage was detected in the comet assay using bone marrow cells of Swiss albino mice as well (7-35 mg kg^{-1} aspartame for 18 h) (Bandyopadhyay et al., 2008). Although aspartame has been extensively investigated for its genotoxicity, there are conflicting results.

The use of artificial sweeteners is a contentious subject due to the view that they may be linked to direct or indirect induction of genotoxic and carcinogenic risks. Little is known about the mechanism by which sweeteners and their metabolites induce DNA breaks. It has been described that the mechanisms of genotoxicity caused by artificial sweeteners, including sorbitol and aspartame, may be DNA single and double-strand breaks, DNA inserts, DNA-DNA, and DNA-protein crosslinks. It has been emphasized that the reason for DNA damage triggered by these sweeteners is the irreversible damage caused by free radicals (reactive oxygen species=ROS) in proteins involved in DNA replication, repair, recombination, and transcription (Lin et al., 2007; Findikli and Turkoglu, 2014).

XYL was included in the "ADI Not Specified" group by JECFA and "REG - Food additives for which a petition has been filed and a regulation issued" group by the FDA. The result of the present investigation determined that XYL has a genotoxic potential only at high concentrations *in vitro* conditions. However, it did not show a genotoxic effect at low concentrations, and this supports the mentioned international organizations.

In conclusion, the results of the present study revealed that XYL has no genotoxic effect at low concentrations in all the four different assays applied. Considering that only high concentrations can cause damage, high doses and excessive use should be avoided, similarly for almost all chemicals.

ACKNOWLEDGMENT

This study is a section of the Ph.D. thesis of EAY.

Contribution of the Authors as Summary

The study design and experimental process were carried out by EAY and DY, and the data were evaluated. FU contributed to comet testing and analysis. All authors contributed to the preparation of the article.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

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